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14. ABSTRACT <p>The radiation therapy (RT) has proven to be effective at increasing survival of men with prostate cancer. However, the results are far from optimal, with 30-40% of men with intermediate to high risk prostate cancer failing within 5 years. We have been investigating agents that have the potential to enhance the cell killing effects of one or both of these treatments. NS-123 is a drug that we have identified as having such potential.</p> <p>The objective of any combination of therapeutic agents is to achieve an improved therapeutic gain. The therapeutic gain is a function of both the tumor and normal tissue response. There is no universally accepted measure of a therapeutic result: lifespan, duration of remission, quality of life are all important and reflect different facets of the total result. When therapies are compared, it is necessary to show that one treatment controls the disease better than another for a similar level of toxicity. We recently reported the results of preclinical studies on a novel radiosensitizer, 4'-bromo-3'-nitropropiphenone (NS-123) that we identified using a cell-based, high-throughput screening method. In these studies, NS-123 radiosensitized human lung adenocarcinoma, colon adenocarcinoma, and glioma cells. Recently, we have demonstrated that NS-123 also radiosensitizes prostate cancer cells. Importantly, NS-123 appears to be a 'true' radiosensitizer as no overt toxicity was seen in any of the normal tissue models that we studied. Investigations into the mechanisms responsible for this radiosensitization suggest that NS-123 inhibits the DNA repair pathways, possible as a result of some upstream inhibition within the phosphatidylinositol-3-kinase/Akt pathway. NS-123 appears to sensitize prostate cancer cells with only a short exposure of 1 hr. Animal studies with daily treatment (50 mg/kg) showed no toxicity. Studies investigating in vivo radiosensitization have been initiated.</p>					
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• INTRODUCTION:

We reported the results of preclinical studies on a novel radiosensitizer, 4'-bromo-3'-nitropropionophenone (NS-123) that we identified using a cell-based, high-throughput screening method.[1] In these studies, NS-123 radiosensitized human lung adenocarcinoma, colon adenocarcinoma, and glioma cells. As part of this project, we have also found that **NS-123 radiosensitizes prostate cancer cells**. Importantly, NS-123 appears to be a 'true' radiosensitizer as no overt toxicity was seen in any of the normal tissue models that we studied. Preclinical investigation of NS-123 has formed the basis for this research proposal. This final report represents work that his team has collectively put together over the time period of the grant.

• BODY:

The proposed experiments are to determine the efficacy of NS-123 as a radiosensitizing agent in the treatment of prostate cancer as well as providing a better understanding of the molecular mechanisms responsible for the control prostate cancer. The body of work consists of a series of *in vitro* and *in vivo* experiments to determine the therapeutic mechanism of NS-123 and if it is worthy of consideration for future research.

Specific Aim 1: Maximize the therapeutic gain obtained by combining NS-123 with RT±AD in prostate cancer cells *in vitro*.

The main goal of this Aim is to determine the optimal *in vitro* dose enhancement ratio (DER) that can be obtained by combining NS-123 with radiotherapy (RT) in prostate cancer cells. To complete this endpoint, clonogenics were performed with multiple time points with respect to drug administration and subsequent RT. The ability of NS-123 to act as a radiosensitizer was planned to be investigated by clonogenic experiments in LNCaP, PC3 and LNCaP-Res prostate cancer cell. However, we did not find any radiosensitivity in LNCaP or LNCaP-Res prostate cells. Therefore, we chose decided to test the radiosensitivity in another human prostate cancer cell line, DU145. Here we were able to identify radiosensitivity by NS-123. These clonogenic experiments were designed to vary both the incubation time prior to irradiation (Pre-IR) and also the time post-irradiation (Post-IR) to determine which condition(s) is most effective at killing the cancer cells based on the Dose Enhancement Ratio (DER) at Survival Fractions of 0.1 and/or 0.01. The higher the DER, the more radiosensitivity observed. A summary of the results is presented in Table 1.

Table 1. Results of Clonogenic Assays (average of at least 2 experiments)					
Cell Line	Pre-IR time (hr)	Post-IR time (hr)	NS-123 (μM)	DER at 0.1	DER at 0.01
DU145	1.0	0.0	20	1.37	
DU145	1.0	0.0	30	1.66	
DU145	1.0	4.0	20	1.81	
DU145	1.0	4.0	30	2.16	
DU145	1.0	24.0	20	1.55	
DU145	1.0	24.0	30	1.83	
DU145	4.0	0.0	20	1.26	
DU145	4.0	0.0	30	1.59	
DU145	4.0	4.0	20	1.24	
DU145	4.0	4.0	30	1.86	
DU145	16.0	0.0	20	1.06	
DU145	16.0	0.0	30	1.07	
DU145	16.0	4.0	20	1.03	
DU145	16.0	4.0	30	1.14	
DU145	24.0	0.0	20	1.02	
DU145	24.0	0.0	30	1.09	
PC3	1.0	0.0	20	1.10	1.03
PC3	1.0	0.0	30	0.95	0.98
PC3	1.0	4.0	20	1.28	1.34
PC3	1.0	4.0	30	1.27	1.29
PC3	4.0	0.0	20	1.05	1.05
PC3	4.0	0.0	30	1.27	1.12
PC3	4.0	4.0	20	1.15	
PC3	4.0	4.0	30	1.05	
PC3	16.0	0.0	20	0.99	0.99
PC3	16.0	0.0	30	1.00	0.96
PC3	16.0	4.0	20	1.06	1.02
PC3	16.0	4.0	30	1.02	0.98
PC3	24.0	0.0	20	0.93	0.91
PC3	24.0	0.0	30	0.91	
PC3	24.0	4.0	20	0.87	
PC3	24.0	4.0	30	0.76	

The lack of radiosensitivity in LNCaP and LNCaP-Res was an unanticipated negative result. Therefore we could not use these cell lines in further experiments and had to focus our effort on the PC3 and DU145 cancer cell lines.

Our *in vitro* studies allowed us to clarify another important issue. Some of the clonogenic assays performed with the varying time we failed to identify radiosensitization, especially when we were using 30 μ M NS-123. We reviewed these experiments and determined that the lack of radiosensitization was function of decreased survival at 0 Gy. NS-123 was functioning more as a cytotoxic agent than as a radiosensitizer. As a result, we investigated lower concentrations of NS-123. Representative plates are presented in Figure 1, below.

Figure 1

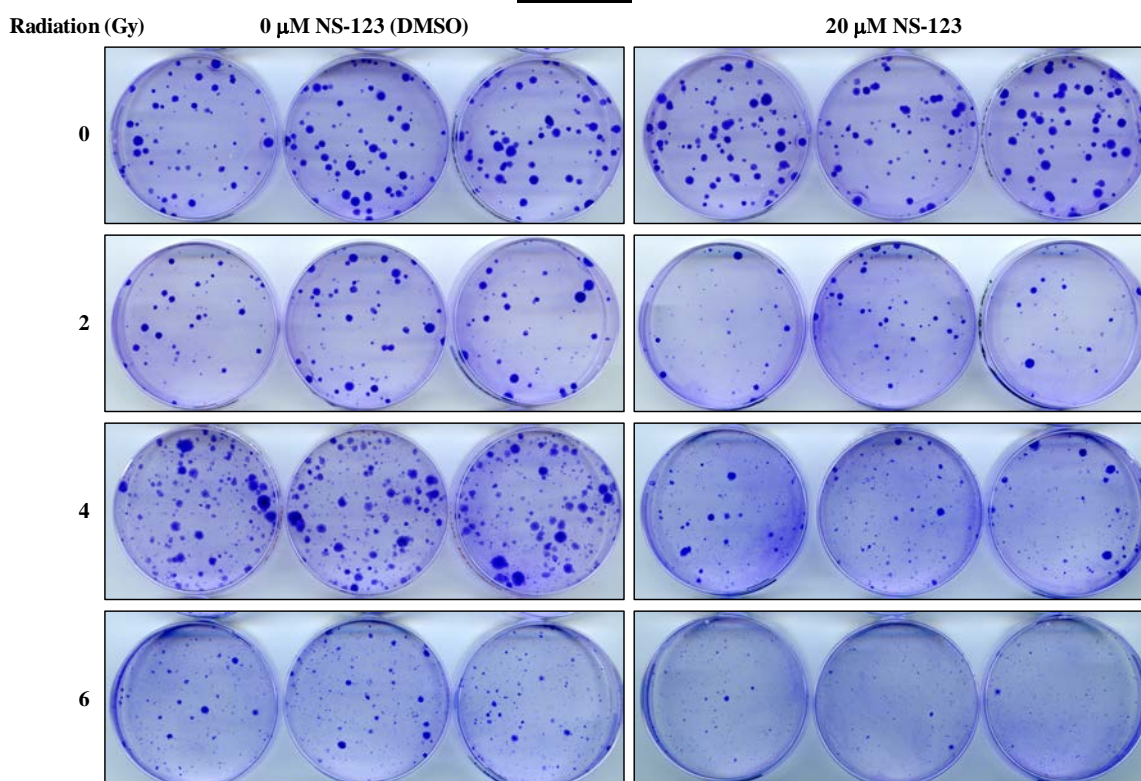


Figure 1. PC3 cells treated with NS-123 for 1 hr, irradiated with a 4 hr recovery period. Cells were trypsinized and plated for colony formation. Colonies were stained with crystal violet after 14 days of growth at 37 °C.

Thus, lower dose of NS-123 are effective at radiosensitizing prostate cancer cells. In Table 2 below, we present the raw data in terms of number of colonies counted. At 0 Gy, no cytotoxicity related to NS-123 was identified.

Table 2. PC3 cells treated with NS-123 for 1 hr, irradiated with a 4 hr recovery period. Cells were trypsinized and plated for colony formation. Colonies were stained with crystal violet after 14 days of growth at 37 °C.

Radiation (Gy)	0 μ M NS-123 (DMSO)		20 μ M NS-123	
	# colonies (avg. 3 plates)	# cells plated	# colonies (avg. 3 plates)	# cells plated
0	82.0	200	85.3	200
2	58.3	500	33.0	500
4	135.0	6,000	42.7	6,000
6	39.3	10,000	8.0	25,000

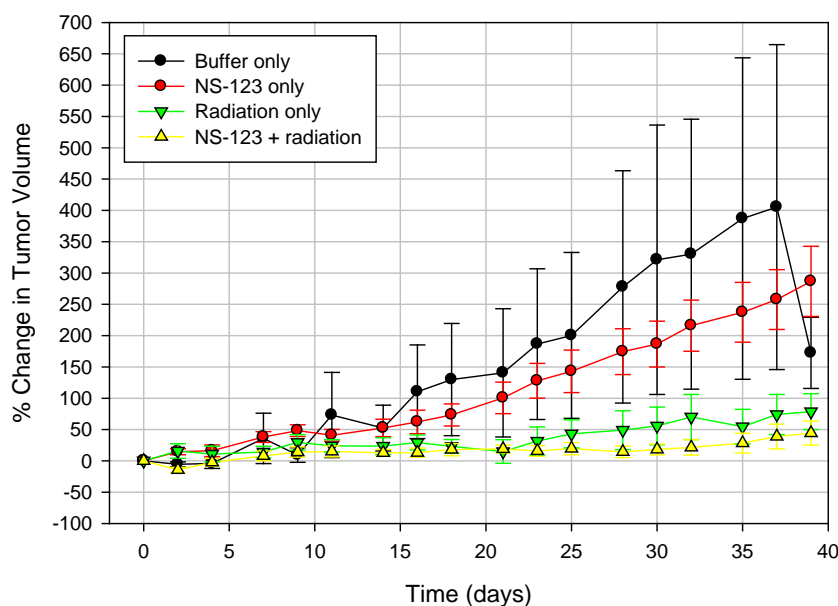
NS-123 (both at 20 and 30 μ M) had more effectiveness as a radiosensitizer in the DU145 cell line compared to PC3. In the DU145 experiments, NS-123 best promoted radiosensitivity when the pre-IR time was short (4 hours or less). The post-IR time did not appear to be a factor. Though not as dramatic, the same trend was observed in the PC3 clonogenic experiments. The significance of these results relevant to our proposed animal studies is that we will administer NS-123 one hour prior to the planned irradiation.

Specific Aim 2: Determine if NS-123 can be integrated into current prostate cancer treatment paradigms to produce an increase in the therapeutic gain *in vivo*.

After obtaining IACUC approval for animal studies, we completed a toxicology study of NS-123. Three male adult nude (nu/nu) mice were injected with 50 mg/kg of NS-123 (dissolved in 10% DMSO, 30% PEG in PBS) and 2 male mice were injected with the buffer only. Mice were injected I.P. daily for 5 consecutive days and maintained for an additional 30 days. All mice survived and no weight loss or toxicity was observed. After the mice were sacrificed, the major organs from each mouse were analyzed by the University of Miami Pathology staff and were found to be within normal limits.

We performed *in vivo* studies to investigating the radiosensitization of NS-123 in male adult nude (nu/nu) mice with implanted PC3 tumor cells. In this aim, we only investigated the PC3 tumor model; we did not investigate the LNCaP tumor model because of the results from Aim 1. Subcutaneous tumors were established by injecting 5×10^5 PC3 cells in the flanks of nude male mice. Tumors were allowed to grow and the mice were randomized into four experimental groups. These groups were: Buffer alone, NS-123 alone, Radiation alone (Buffer + RT), and NS-123 + RT; 9-10 mice were in each group. Treatments were started when tumor volumes were approximately 50 mm³ and was for five consecutive days (first day of treatment = Day 0 in Figure 2). Based on results of **Aim 1**, 50 mg/kg of NS-123 (or Buffer) was administered 1 hour prior to irradiation of the tumors. The mice were restrained and shielded such that only the tumor was irradiated. Tumor size was assessed with caliper measurements three times/week, and tumor volume calculated from the formula $TV = \frac{\pi}{6} \times 1.69 \times (L \times W)$ raised to the 1.5 power.³ As Figure 2 shows, In this experiment, NS-123 showed some activity but did not reveal any definite radiosensitization when the NS-123 + RT data was compared to the Buffer + RT data.

Figure 2. Results of NS-123 investigated in Xenograft tumor model.



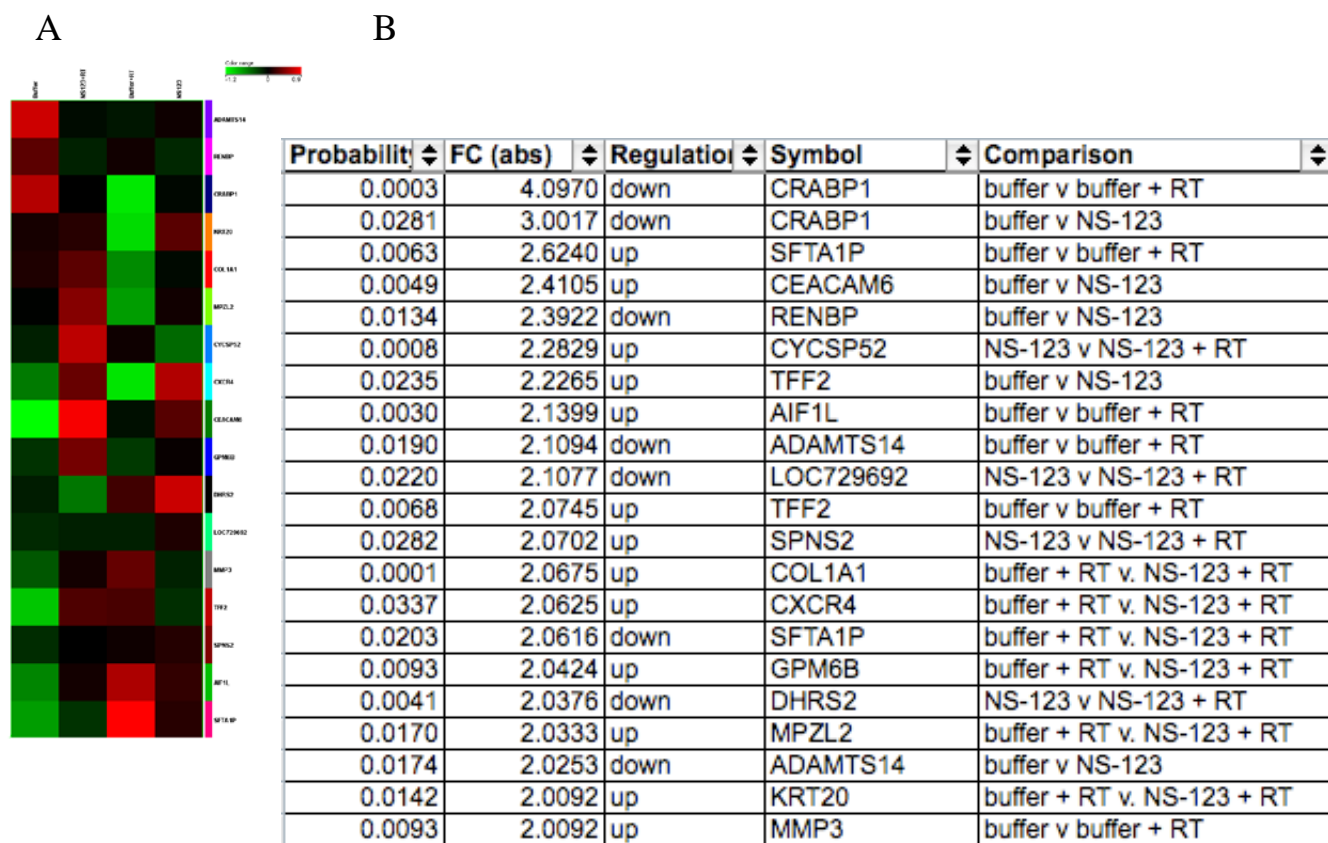
Mice were sacrificed by carbon dioxide asphyxiation between 25-49 days post-treatment and the tumors removed within 15 minutes and placed in RNA Later (Ambion) and stored at 4 °C for ~ 7 days, and then transferred to -20 °C until the RNA was extracted. RNA extraction was done using Qiagen's miRNAeasy Kit. All the RNAs were extracted within 5 days of each other. The University of Miami Oncogenomics Core Facility (OCF, directed by Dr. Toumy Guettouche) performed all of the following RNA analyses. The extracted RNA was quality checked and quantified and used for the

microarray analysis. The RNAs were analyzed either on Illumina's HT-12 chip (RNAs of high quality) or Illumina's DASL chip (RNAs of lower, but acceptable quality). Three tumors from each treatment group were also analyzed on Illumina's mouse WG-6 chip. This mouse microarray assay was done to ensure that the experimental treatments themselves did not significantly alter the expression of any mice genes, a concern considering the experimental design. The microarray data was analyzed by Dr. Biju Isaac from the University of Miami's Bioinformatics and Biostatistics Core.

For each microarray assay (chip), the data was analyzed as a group in that all the samples and the data points from each treatment group was compiled and analyzed as one group. In looking at the concordance of each individual sample within a treatment group, in general it was very good; however, on occasion there was one sample within each group that did not agree as well. This method of analyzing the data was chosen to give us an overview of the data and if there was something interesting, then we could go back and more closely review the data from each individual mouse tumor.

About half the genes were up regulated, the other half down regulated and the fold change in these genes was usually between 2-6 fold. A few genes had several hundred fold change. We will need to look at the individual data from each tumor specimen to see if this fold change is truly representative of the group or due to an outlier. In addition, there were no genes that consistently showed up in all groups (or comparisons) as being up or down regulated. At most a gene was shown to be affected in only one or two comparisons groups. Because there was not a consistent pattern of genes being up/down regulated, we conclude from the WG-6 mouse microarray experiment that the process of generating tumors and our treatment regime did not overtly affect mouse gene expression.

Figure 3: Gene expression data from the human microarray



In the above Figure 3, we show a representative analysis of our microarray data. On the left, a heat map for the 17 genes were shown to have a FC > 2.0 at P < 0.05 and the change of expression is shown in each of the four treatment groups (Buffer, NS-123+RT, Buffer+RT, NS-123). The genes identified are varied with respect to their function and pathways. On the right is listing of the genes whose expression was up/down regulated by 2-fold or more.

The main goal of this mouse experiment was to determine if treating mice with NS-123 prior to radiation would affect tumor growth, specifically act as a radiosensitizer *in vivo* and retard tumor growth even more so than radiation alone. As the graph displaying the change in tumor volume indicates, the tumors in mice receiving radiation (+/- NS-123) grew slower than those in the control groups (mice not receiving radiation +/- NS-123). However, the change in tumor volume between the NS-123 + RT and the RT only groups was very similar and as such, we cannot conclude that NS-123 is acting as a radiosensitizer in this experimental set-up and with the number of mice (9) analyzed in each group.

- **KEY RESEARCH ACCOMPLISHMENTS:**

- NS-123 radiosensitizes prostate cancer cells with a variety of treatment schedules *in vitro*.
- NS-123 at a dosage of 50 mg/kg of body weight given I.P. for five consecutive days did not show any overt toxicity to mice and their major organ systems.
- NS-123 does not appear to radiosensitize prostate cancer cells *in vivo*.

- **REPORTABLE OUTCOMES:**

- **B.E. Lally**, C. Koumenis, Z. Mu, J.R. Testa, A. Pollack. Preclinical Studies of a Novel Small Molecule Radiosensitizer of Prostate Cancer. *International Journal of Radiation Oncology, Biology, & Physics*. Volume 75, Issue 3, Supplement 1, *Page S561*. Proceedings of the American Society for Radiation Oncology 51st Annual Meeting, 51st Annual Meeting of the American Society for Radiation Oncology McCormick Place West, Chicago, IL November 1-5, 2009
- An abstract entitled "Preclinical testing of a novel small molecule radiosensitizer of prostate cancer cells" was presented at the May 2011 Sylvester Cancer Center Annual Retreat.
- An abstract entitled "Preclinical testing of a novel small molecule radiosensitizer of prostate cancer cells" presented at the The Innovative Minds in Prostate Cancer Today (IMPACT) conference held in Orlando, Florida, on March 9-12, 2011.

- **CONCLUSION:**

In this series of experiments, we did not find the needed evidence to support further investigation of NS-123 in clinical trials to treat prostate cancer in humans. The value of NS-123 may exist in other disease sites or with some chemical modification.

- **REFERENCES:** List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

1. Lally, B.E., et al., *Identification and biological evaluation of a novel and potent small molecule radiation sensitizer via an unbiased screen of a chemical library*. *Cancer Res.*, 2007. **67**(18): p. 8791-8799.
2. Morgan, P.B., et al., *Radiation dose and late failures in prostate cancer*. *Int J Radiat Oncol Biol Phys*, 2007. **67**(4): p. 1074-81.
3. Feldman, J.P., Goldwasser, R., Schlomo, M, Schwartz, J, and Orion, I. *A Mathematical model for tumor volume evaluation using two-dimensions*. *J Applied Quant Methods*, 2009. **4**(4): 455-462.

- **APPENDICES:**

Included: Pathology NS-123 mice toxicity.

Accession # 11-7082

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Accession Date: 06/06/2011

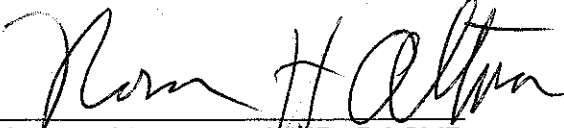
Investigator: Rad Oncology/ Lally
Animal ID: A1, A2, A3 ,A4, & A5

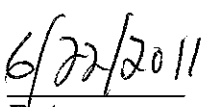
Date to Compath lab: 06/206/2011

Comments:

Enclosed is the histological evaluation of tissues from the 5 mice submitted.
All were Within Normal Limits.

A1-1	Brain	Within Normal Limits
A1-2	Sternum & Femur	Within Normal Limits
A1-3	Lung, Heart, Liver w/ Gall Bladder	Within Normal Limits
A1-4	Stomach, Kidney w/ Adrenal, Bladder, Skeletal Muscle	Within Normal Limits
A1-5	Intestinal Roll	Within Normal Limits
A2-1	Brain	Within Normal Limits
A2-2	Sternum & Femur	Within Normal Limits
A2-3	Lung, Heart, Liver w/ Gall Bladder	Within Normal Limits
A2-4	Stomach, Kidney w/ Adrenal, Bladder, Skeletal Muscle	Within Normal Limits
A2-5	Intestinal Roll	Within Normal Limits
A3-1	Brain	Within Normal Limits
A3-2	Sternum & Femur	Within Normal Limits
A3-3	Lung, Heart, Liver w/ Gall Bladder	Within Normal Limits
A3-4	Stomach, Kidney w/ Adrenal, Bladder, Skeletal Muscle	Within Normal Limits
A3-5	Intestinal Roll	Within Normal Limits
A4-1	Brain	Within Normal Limits
A4-2	Sternum & Femur	Within Normal Limits
A4-3	Lung, Heart, Liver w /Gall Bladder	Within Normal Limits
A4-4	Stomach, Kidney w/ Adrenal, Bladder, Skeletal Muscle	Within Normal Limits
A4-5	Intestinal Roll	Within Normal Limits
A5-1	Brain	Within Normal Limits
A5-2	Sternum & Femur	Within Normal Limits
A5-3	Lung, Heart, Liver w/ Gall Bladder	Within Normal Limits
A5-4	Stomach, Kidney w/ Adrenal, Bladder, Skeletal Muscle	Within Normal Limits
A5-5	Intestinal Roll	Within Normal Limits


Norman H. Altman, VMD, DACVP


Date